Chapter 10

General discussion and summary

Regulation of airway smooth muscle phenotype and function by extracellular matrix proteins

Shedding of the epithelium, goblet cell hyperplasia, increased blood vessel formation, enhanced deposition of extracellular matrix (ECM) proteins and increased airway smooth muscle (ASM) mass are characteristic aspects of airway wall remodelling in asthma [1-3]. Increased ASM mass may be explained by ASM hyperplasia as well as by hypertrophy [4] and is considered to be a major factor contributing to airway hyperresponsiveness and decline in lung function in asthmatics [5,6]. In keeping with hyperplasia, ASM cells, in contrast to skeletal myocytes and cardiomyocytes [7,8], retain their ability to enter the cell-cycle and change their phenotype (phenotypic plasticity) [9]. *In vitro*, exposure of ASM cells and tissue to serum and various growth factors results in a switch from a contractile to a proliferative phenotype. This phenotype is characterized by increased expression of proliferative markers such as Ki67, increased proliferation, decreased expression of contractile marker proteins like smooth muscle α-actin (*sm*-α-actin), calponin and smooth muscle myosin heavy chain (*sm*-MHC), and decreased contractile function [10,11]. ASM phenotype switching is reversible, as indicated by the observation that removal of mitogenic stimuli, for example by serum deprivation of the culture medium in the presence of insulin or TGF-β results in the reintroduction of a contractile ASM phenotype, associated with increased contractile protein expression and increased contractile function [12,13].

Increased ECM deposition is another characteristic feature of airway remodelling in asthma. Studies on the nature of ECM changes in the airway wall of asthmatics have revealed increased subepithelial deposition of collagens I, III and V, fibronectin, tenascin, hyaluronan, versican, biglycan, lumican and several laminin chains (α2, α3, α5, β1, β2 and γ1) [14-21]. Patchy staining for laminin α1 chains has also been observed in the airways of allergic asthmatics, whereas no staining was observed in the airways of non-allergic asthmatics or healthy subjects [21]. On the other hand, expression of collagen IV, decorin and elastin in the airway wall of asthmatic patients is decreased [22,23]. The composition of ECM in the microenvironment surrounding the asthmatic ASM cell is changed as well, and is characterized by increased deposition of collagen I, fibronectin, hyaluronan, versican, biglycan, lumican and elastic fibres [24-26]. *In vitro,* ASM cells produce a variety of ECM proteins, including collagens, fibronectin, laminins, perlecan, elastin, thrombospondin, versican, decorin, chondroitin sulphate and hyaluronan [27-30]. ECM synthesis by asthmatic ASM cells is changed compared to that of healthy cells and is characterized by increased production of collagen I, perlecan and fibronectin, and decreased production of laminin α1, chondroitin sulphate, collagen IV and hyaluronan [27,30,31]. This may contribute to the changed ECM microenvironment of the ASM cells and has been shown to have an impact on ASM function, potentially underlying intrinsic differences observed between healthy and asthmatic ASM cells (**Chapter 2**).

Thus, ASM cells obtained from asthmatics proliferate faster than those obtained from healthy subjects [32]. whereas proliferation of healthy ASM cells is increased when cultured on a matrix secreted by asthmatic ASM cells and *vice versa* [27]. Similarly, increased production of eotaxin by asthmatic ASM cells, a feature of increased synthetic function, is also dependent on the ECM proteins produced by these cells [31].

Recent reports have demonstrated that changes in the ECM environment of ASM cells may induce phenotype switching as well. Thus, culturing of human ASM cells on the ECM proteins fibronectin and collagen I increased proliferative responses to the growth factors thrombin and platelet-derived growth factor (PDGF), whereas the PDGF-induced reduction in protein expression of *sm*-α-actin, calponin and *sm*-MHC was further enhanced by culturing the cells on these ECM proteins [33]. On the other hand, culturing ASM cells on laminin-111 or matrigel decreased the proliferative responses and maintained contractile protein expression in the presence of growth factors [33].

Altered deposition of ECM proteins in the airway wall could also alter mechanical properties of the ASM by phenotypic modulation, as has been observed previously for growth factors [11]. Thus, changes in proliferation of cultured bovine tracheal smooth muscle (BTSM) cells in response to peptide growth factors were found to be tightly correlated with growth factor-induced changes in contractility of intact strips, in which endogenous ECM constituents and cell-to-cell contacts are still intact [11]. Using the same approach, we now investigated the functional impact of ASM exposure to fibronectin, collagen I and laminin-111 (**Chapter 3**). In this study, it was demonstrated that culturing BTSM cells on fibronectin and collagen I matrices increased the proliferation of these cells, whereas prolonged exposure (4 days) of BTSM strips to these ECM proteins attenuated the maximal contractile responses to the receptor-dependent agonist methacholine and the receptor-independent stimulus KCl. These changes were associated with decreased protein expression of *sm*-α-actin, calponin and *sm*-MHC in the strips. Collectively, these effects were similar to the phenotypic changes observed for PDGF. Moreover, culturing of ASM cells on fibronectin or collagen I augmented PDGF-induced proliferation in an additive fashion; however, without additional effects on contractility or contractile protein expression. The fibronectin-induced depression of contractility was blocked by the integrin antagonist Arg-Gly-Asp-Ser (RGDS), but not by its negative control Gly-Arg-Ala-Asp-Ser-Pro (GRADSP), indicating that interaction of the ECM protein with its integrin receptors was required. Laminin-111 did not affect contractility on its own, but reduced PDGF-induced hypocontractility. Similarly, PDGF-induced proliferation was reduced when cells were cultured on laminin-111. Strong correlations were observed between ECM-induced changes in BTSM strip contractility and *sm*-α-actin, calponin and *sm*-MHC expression, indicating that changes in contractile protein expression underlie the changes in contractile force. As observed for peptide growth factors [11], strong inverse

correlations between altered contractility and mitogenic responses were observed as well, suggesting that the changes in contractility were indeed due to ASM phenotype switching. Moreover, our findings clearly suggest that ECM proteins differentially affect ASM phenotype and function.

To determine the impact of phenotype switching on contractile function in human ASM, a similar approach was used by Moir *et al* [34]. In this study, exposure of intact human bronchiole ring segments to serum for 3 or 6 days did not affect ASM area or protein expression of *sm*-α-actin and *sm*-MHC. However, 6 days of culturing in the presence of serum reduced carbachol-, histamine- and KCl-induced contractions and decreased calponin expression, suggesting that the ASM phenotype - at least to some extent - had switched to a hypocontractile state [34]. In **Chapter 4**, we addressed the impact of ECM- and growth factor-induced phenotype switching on the function of intact human ASM. In this chapter, it was demonstrated that prolonged exposure (4 days) of human tracheal smooth muscle (HTSM) strips to collagen I or PDGF decreased the maximal contractions induced by methacholine or KCl, which was associated with decreased expression of the contractile proteins *sm*-α-actin and *sm*-MHC. In addition, both collagen I and PDGF increased proliferation of cultured primary HTSM cells. As observed for BTSM (**Chapter 3**), it was found that culturing of HTSM cells on collagen I additively increased the proliferative responses by PDGF, whereas no additional effects of combined treatment were observed on contractility or contractile protein expression. Moreover, the results presented in **Chapter 6** indicate that ECM-induced phenotype switching of human ASM cells may occur not only after exposure to collagen I, but also after exposure to fibronectin, as culturing of these cells on fibronectin increased their proliferation as well. In conclusion, these results indicate that ECM- and growth-factor-induced phenotype modulation is of relevance to human ASM function. Interestingly, our findings also indicate that BTSM is a representative experimental model for human ASM phenotype plasticity.

In addition to increased ASM mass, increased expression of contractile marker proteins has been reported in asthmatic ASM as well (**Chapter 2**) [35]. *In vitro,* expression of contractile marker proteins is increased by serum deprivation, which is further augmented in the presence of insulin or TGF-β [12,13]. Studies on the role of ECM proteins in this process, showed that expression of laminin α2, β1 and γ1 chains is increased during serum deprivation [36]. Increased laminin expression was required for ASM maturation as the laminin β1 chain competing peptide Tyr-Ile-Gly-Ser-Arg (YIGSR) and the RGD containing peptide Gly-Arg-Gly-Asp-Ser-Pro (GRGDSP) both inhibited the increase in contractile protein expression [36]. Moreover, ASM cells increase expression of the laminin binding integrins α3, α6 and α7 during maturation, of which the α7 integrin was found essential for ASM maturation [37].

Exposure of BTSM strips and cells to insulin results in the induction of a hypercontractile, hypoproliferative phenotype, characterized by increased

contractile protein expression, increased contractile responses and decreased proliferation in response to growth factors [13,38]. The role laminins in the induction of a hypercontractile, hypoproliferative ASM phenotype by insulin are described in **Chapter 5**. The results presented in this chapter demonstrate that insulin-induced hypercontractility is inhibited by the laminin competing peptides YIGSR and RGDS, while no effects were observed for GRADSP. In addition, increased expression of the contractile protein *sm*-MHC and decreased proliferative responses after insulin treatment were normalized by YIGSR as well. The insulin-induced increase in BTSM contractility was associated with increased protein expression of laminin α2, β1 and γ1 chains, suggesting that increased laminin-211 expression is required for the induction of a hypercontractile ASM phenotype by insulin. As observed previously for contractile protein accumulation [13], PI3-kinase and Rho kinase signalling pathways were required for the insulin-induced increase in laminin expression and hypercontractility. Collectively, these results indicate a critical role for laminins in the induction of a hypercontractile, hypoproliferative ASM phenotype by prolonged insulin exposure. Increased laminin expression in the airways of asthmatic patients could be involved in the increased contractility and contractile protein expression of asthmatic ASM. Moreover, the results may have important implications for the use of inhaled insulin formulations in diabetes mellitus.

As also indicated above, ASM cells and ECM proteins communicate with each other mainly via integrins, a group of heterodimeric, transmembrane glycoproteins [39]. *In vitro* studies have indicated that integrins are importantly involved in ECM-induced changes in ASM cell adhesion, maturation, synthetic function, survival and proliferation [23,36]. Various integrins have been implicated in ASM proliferation. Thus, the collagen-binding α2β1 integrin as well as the fibronectin-binding α4β1 and α5β1 integrins were shown to be required for the enhanced growth factor-induced proliferation on collagen I and fibronectin [40]. The α5β1 integrin has also been found to be important in serum-induced proliferation of both nonasthmatic and asthmatic ASM [41]. Moreover, increased eotaxin secretion by asthmatic ASM largely required the α5β1 integrin [31].

Although integrins have been shown to modulate ASM function *in vitro,* the importance these ECM receptors in allergen-induced ASM remodelling *in vivo* has not yet been explored. Using a guinea pig model of chronic allergic asthma [42], the contribution of integrins and ECM proteins to allergen-induced airway remodelling *in vivo* was investigated using the integrin-blocking peptide RGDS, which contains the RGD binding motif present not only in fibronectin, but also in collagens and laminins and which blocks the binding of these ECM proteins to multiple integrins [43,44], including the α3β1, α5β1, αvβ1 and αvβ3 integrins which are expressed by ASM cells in culture [40,45]. The results described in **Chapter 6** demonstrate that RGD-binding integrins and their endogenous agonists are important contributors to ASM remodelling in chronic allergic asthma. It was shown that topical treatment of the airways with RGDS

inhibited ASM hyperplasia, pulmonary expression of the proliferative marker proliferating cell nuclear antigen (PCNA), increased contractile protein expression and ASM hypercontractility induced by repeated allergen challenge once a weekly for 12 weeks. No effects were observed for the negative control peptide GRADSP. RGDS did not affect allergen-induced fibrosis or inflammation, which may suggest that the peptide directly influenced the ASM cell. Fully in line, it was found that proliferative responses induced by collagen I, fibronectin, serum and PDGF in human ASM cells required signalling via RGD-sensitive integrins, particularly of the α5β1 subtype. In line with the results described in **Chapter 5**, RGDS also dose-dependently inhibited *sm*-α-actin accumulation induced by serum deprivation in the presence of insulin. Collectively, these findings for the first time indicate that integrins modulate ASM remodelling in allergic asthma, which can be inhibited by a small peptide containing the RGD motif.

No effects of RGDS were observed in saline-challenged guinea pigs (**Chapter 6**), suggesting that integrin activation occurs only under pathological conditions. In allergen-challenged animals, integrin activation may result from synthesis of new ECM proteins, recruitment of plasma-derived ECM proteins to the ASM layer or alterations in the existing ECM, exposing the matricryptic integrin binding sites which are normally hidden within the proteins [46,47]. In support of such a notion, denatured monomeric collagen I, but not fibrillar collagen I, increased growth factor-induced proliferation of human ASM, suggesting that changes in the configuration of the ECM are required for the induction of proliferative signals [40]. Similarly, no effects of RGDS or the function-blocking antibodies were observed on ASM cell numbers (**Chapter 6**) or BTSM strip contractility (**Chapter 3**) under unstimulated (control) conditions, suggesting that exposure of RGD-binding integrins to their recognition sites within the ECM proteins is limited under these conditions.

Previous studies [36] and the results described in **Chapter 5** suggest that laminins are important in a shift of the ASM to a hypercontractile, hypoproliferative phenotype. Therefore, the effects of the specific laminin-competing peptide YIGSR on airway remodelling were also investigated in our guinea pig model of chronic allergic asthma (**Chapter 7**). Surprisingly, topical administration of YIGSR in the airways *attenuated* allergen-induced ASM hyperplasia and pulmonary PCNA expression. Treatment with YIGSR also *increased* pulmonary *sm*-MHC expression and ASM contractility, both in saline and allergen-challenged animals, suggesting that treatment with the peptide *increased* rather than *decreased* laminin function. *In vitro*, culturing of human ASM cells on immobilized YIGSR concentration-dependently reduced PDGF-induced proliferation of the cells to a similar extent as culturing of the cells on laminin-111. Remarkably, the effects of both immobilized YIGSR and laminin were antagonized by soluble YIGSR. Collectively, these results indicate that *in vivo* treatment with YIGSR mimics rather than inhibits laminin function, which

seems to depend on the physicochemical microenvironment of the peptide. The mechanisms underlying these differential effects are currently unknown.

Collectively, the findings presented in **Chapters 3-7** point to an important functional role of cell – matrix interactions in the regulation of ASM phenotype. Changes in the extracellular microenvironment surrounding the asthmatic ASM cells may therefore importantly contribute to the increased contractile, proliferative and synthetic characteristics of these cells. The ASM itself may be importantly involved in creating an ECM environment that supports these abnormal ASM functions.

Extracellular matrix-induced signal transduction in airway smooth muscle

In several cell types it has been established that specific integrin – ECM interactions result in the activation of various intracellular signalling cascades required for changes in proliferation, migration, differentiation and survival induced by ECM proteins [48]. Various integrins activate focal adhesion kinase (FAK), which then subsequently activates downstream signalling pathways, including PI3-kinase- and MAPK-dependent pathways [48]. The results described in **Chapter 8** indicate an important role for the activation of FAK and downstream signalling pathways in the induction of a proliferative, hypocontractile ASM phenotype by collagen I. Using BTSM cells *in vitro*, it was shown that FAK is activated during adhesion to an uncoated plastic matrix and to a collagen I matrix, without differences between the two conditions. Activation of FAK was required for cell adhesion, as adhesion was inhibited by overexpression of the FAK deletion mutants FAT (derived from the Focal Adhesion Targeting (FAT) domain of FAK) and FRNK (FAK-Related Non-Kinase), which both inhibit FAK localization to the focal adhesions and subsequent FAK activation [49,50]. FAK activation was further increased by culturing on collagen I for 2-4 days, but not by culturing on plastic. The delayed activation suggests that FAK is not directly activated by collagen I, but that additional secondary processes may be required. In support of this notion, monomeric collagen I has been shown to increase the expression of other ECM components, including fibronectin, in vascular smooth muscle cells [51]. Moreover, the collagen I-induced increase in basal (**Chapter 6**) and growth factor-dependent [40] proliferation required interaction with the fibronectin-binding integrin α5β1.

In our experiments, collagen I increased BTSM cell proliferation in a concentration- and time-dependent fashion, which was inhibited by overexpression of FAT and FRNK. Pharmacological inhibition of the Src, MEK, PI3-kinase and p38 MAPK signalling cascades inhibited collagen I-induced proliferation and hypocontractility, suggesting that activation of downstream

signalling pathways of FAK is required for collagen I-induced phenotype modulation. Next to its role in collagen I-induced ASM phenotype modulation, FAK has also been shown to be directly involved in contractile responses, as indicated by the observation that downregulation of FAK in HTSM strips decreased tension development, myosin light chain phosphorylation and calcium signalling in response to acetylcholine and KCl [52], suggesting that FAK may pose not only a target in the treatment of persistent but also in acute airway hyperresponsiveness.

Effects on glucocorticosteroids and β_2-adrenoceptor agonists on ASM phenotype switching

Glucocorticosteroids and β_2-adrenoceptor agonists are currently the most effective therapy for asthma control based on their anti-inflammatory and bronchodilating actions, respectively [53,54]. Treatment with glucocorticosteroids has been shown to prevent increases in ASM mass in animal models of asthma [55], suggesting that these drugs also affect airway remodelling. *In vitro*, both glucocorticosteroids and β_2-adrenoceptor agonist have been shown to inhibit ASM proliferation [56-59]. However, anti-mitogenic actions of glucocorticosteroids were shown to be reduced when ASM cells are cultured on collagen I matrices [60-62]. This indicates that increased deposition of collagen I surrounding the ASM may not only increase ASM proliferation, but may also contribute to steroid resistance as observed in severe asthmatics. Clinical studies have indicated that combined treatment with glucocorticosteroids and β_2-agonists results in a better asthma control than monotherapy with each of the drugs [63]. In human lung, glucocorticoids have been shown to increase transcription of the β_2-adrenoceptor gene [64] and in ASM cells, β_2-agonists have been shown to synergistically increase glucocorticosteroid receptor translocation [57]. However, the impact of this synergism on ASM phenotype switching remains to be established. The results described in **Chapter 9** demonstrate that the glucocorticosteroids fluticasone, budesonide and dexamethasone inhibited both PDGF- and collagen I-induced BTSM switching to a proliferative, hypocontractile phenotype, although the collagen I-induced phenotype switch was less sensitive to glucocorticosteroid action. Proliferative responses induced by both mitogens were inhibited by the β_2-agonist fenoterol as well. When applied in 100-fold lower concentrations, that were not or only partially effective, fluticasone and fenoterol synergized to inhibit ASM phenotype switching induced by PDGF and collagen I. As increased ASM mass is likely to be the most important factor of increased airway resistance and AHR in asthma [5,6], these findings suggest that combined treatment may effectively reduce decline in lung function and persistent AHR in chronic asthma. Since not only growth factor-, but also collagen I-induced phenotype switching was inhibited by the combined treatment, it can be envisaged that, in addition to increasing anti-

inflammatory, bronchodilating and anti-proliferative effects [63], normalizing steroid sensitivity by β_2-agonists contributes to the enhanced asthma control by the combination therapy. Collectively, these findings may contribute to the enhanced efficacy of β_2-adrenoceptor agonist/glucocorticosteroid combination therapy in controlling asthma.

Taken together the studies described in this thesis reveal that:

- ECM proteins differentially regulate ASM phenotype and contractile function. Fibronectin and collagen I induce a functionally hypocontractile ASM phenotype, associated with increased proliferative responses, whereas laminin-111 maintains a functionally contractile phenotype and inhibits growth factor-induced proliferation (**Chapters 3 & 6**).
- Collagen I and PDGF induce a proliferative, hypocontractile phenotype in human ASM, which is of relevance to ASM function (**Chapter 4**).
- Insulin-induced laminin α2, β1 and γ1 chain expression, mediated by PI3-kinase- and Rho kinase-dependent signalling pathways, contributes to the induction of a hypercontractile, hypoproliferative ASM phenotype. Increased laminin expression in the airway wall could contribute to the increased ASM contractility and contractile protein expression as observed in asthma (**Chapter 5**).
- Induction of contractile protein and laminin expression in response to insulin may limit the use of inhaled insulin formulations in diabetes mellitus (**Chapter 5**).
- Endogenous activation of RGD-binding integrins modulates allergen-induced ASM remodeling in an animal model of allergic asthma, which can be inhibited by topical application of RGDS, a small peptide containing the RGD motif. Based on these findings, RGD-binding integrins may represent a novel target in the treatment of airway remodeling in asthma (**Chapter 6**).
- The laminin β1 chain-competing peptide YIGSR inhibits the induction of a hypercontractile, hypoproliferative ASM phenotype *in vitro* (**Chapter 5**). By contrast, *in vivo* YIGSR promoted a contractile, hypoproliferative ASM phenotype, which could depend on the physicochemical microenvironment of the peptide (**Chapter 7**).
- Activation of FAK is obligatory for ASM cell adhesion both to plastic and to collagen I matrices and mediates collagen I-induced proliferation of these cells (**Chapter 8**).
- Phenotypic modulation of ASM cells by collagen I is dependent on Src, MEK, p38 MAPK and PI3-kinase, which may be activated downstream of FAK (**Chapter 8**).
- Glucorticosteroids inhibit ASM phenotype switching induced by PDGF and collagen I, the effects of collagen I being more resistant to glucocorticosteroid action. Low doses of glucocorticoids synergize with low

doses of β_2-adrenoceptor agonists to prevent the induction of a proliferative, hypocontractile ASM phenotype, without differences between the two mitogens. This synergism could contribute to enhanced control of asthma by combination therapy (**Chapter 9**).

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